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Ectopic expression of the maize *kn1* gene phenocopies the *Hooded* mutant of barley

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SUMMARY

The homeobox gene, *knotted1*, (*kn1*) is expressed in shoot meristems and is required for maintaining indeterminacy and preventing cellular differentiation. Awns, extensions of the bract-like lemma found in all grass inflorescences, are normally determinate structures. We show that ectopic expression of *kn1* in the barley awn is sufficient to direct the development of ectopic meristems, forming inflorescence-like structures. This homeotic transformation is similar to the phenotype produced by misexpression of the barley *hvknox3* gene, associated with the dominant *Hooded* mutant (Müller, K. J., Romano, N., Gerstner, O., Garcia-Maroto, F., Pozzi, C., Salamini, F. and Rohde, W. (1995) *Nature* 374, 727-730). We suggest that the inverse polarity of the ectopic flowers seen in *Hooded* and transgenic *kn1* plants results from the transformation of the awn into reiterative inflorescence axes. We observed that the protein and mRNA localization of the transgene, driven by a constitutive promoter, is similar to the expression pattern of *hvknox3* in awns of *Hooded* mutants, suggesting posttranscriptional regulation.

Key words: *knotted1*, meristem, homeobox, barley, *Hooded*, phenocopy, ectopic expression, ectopic inflorescence.

INTRODUCTION

Plants elaborate their shoot system through the activity of the shoot apical meristem which forms organs from its flanks. In flowering plants a variety of associations exists between an individual flower and the shoot system. A single flower may arise through modification of an individual vegetative shoot apex or more often a vegetative shoot may be transformed into a flowering shoot or inflorescence that contains several or many individual flowers (Steeves and Sussex, 1989). All grass inflorescences are organized in spikelets; each spikelet containing one or many florets (Fig. 1). The barley spikelet develops as a shortened stem axis, the rachilla, which is attached to the main axis or rachis and bears two overlapping sterile bracts (glumes) in alternating order. The third bract is the lemma which bears a flower in its axil. The lemma terminates in a long pointed extension, an awn, considered to be a modified leaf blade (Dahlgren et al., 1985).

In barley, a gene homologous to the maize *knotted1* (*kn1*) homeobox gene is associated with the *Hooded* locus (Müller et al., 1995). Plants bearing the *Hooded* mutation develop ectopic meristems on the upper lemma and awn. In some genetic backgrounds, the meristem develops into an ectopic floret which contains the normal series of parts: palea, two lodicules, three anthers, and an ovary with two stigmas. Additional ectopic reproductive structures can arise on the awn, with each iteration in inverse polarity to that preceding it (Stebbins and Yagil, 1966). Other parts of the plant are normal.

In this study we focus on the *kn1* homeobox gene family (Vollbrecht et al., 1991) which is expressed in shoot meristems (Smith et al., 1992). *kn1* is required for meristem maintenance, particularly in the inflorescence (Kerstetter et al., 1997). A related gene in *Arabidopsis*, *SHOOTMERISTEMLESS*, is required for initiation and maintenance of the shoot apical meristem (Barton and Poethig, 1993; Long et al., 1996). In this report we show that ectopic expression of maize *kn1* in transgenic barley plants results in phenotypic abnormalities along the awns that resemble the *Hooded* mutant phenotype. We characterize the naturally occurring *Hooded* mutation in three different genetic backgrounds and propose a model to explain the inverse orientation of the ectopic meristems observed in *Hooded* and transgenic *kn1* plants. Surprisingly, the patterns of gene expression of the endogenous *kn1*-like gene in *Hooded* and the *kn1* transgene driven by a constitutive promoter are striking in their similarity.

MATERIALS AND METHODS

Barley lines

Barley (*Hordeum vulgare* L.) spring cultivar Golden Promise was used for stable transformation and for scanning electron microscopy studies. Seeds for Golden Promise, Strip Tease (CI 6837) and the *Hooded* (*KK*) variety, Haybet (Betzes *Hooded*) (CI 16569) were obtained from H. Bockelman, National Small Grains and Potato Germplasm Research Facility, Aberdeen, ID. Haybet was developed

by crossing the *Hooded* variety Strip Tease as a male parent to the recurrent parent Betzes (CI 6398) retaining the *Hooded* phenotype in subsequent back-crosses (Hockett and Bowman, 1990). The *Hooded* stock BGS152, in the background Colsess, was obtained from N. Lapitan, Colorado State University, Fort Collins, CO.

Plant growth conditions

Donor material used for stable transformation was obtained from plants grown in growth chambers under a 16-hour light/8-hour dark period at 12°C and 60-80% humidity. All other barley plants used for morphological and phenotypic analysis were grown in the greenhouse in Berkeley, CA under a 14-hour light/10-hour dark period at 15-18°C; natural and supplemental light levels were maintained at 700-1000 μmol/m^2/second.

Plasmids

One plasmid used for stable transformation was pAHC20 (Christensen and Quail, 1996), which contains *bar*, isolated from *Streptomyces hygroscopicus* (deBlock et al., 1987), under the control of the maize *ubiquitin* (*ubi1*) promoter and first intron (Christensen et al., 1992) with a nopaline synthase 3′ termination sequence (*nos*) from *Agrobacterium tumefaciens* (Christensen and Quail, 1996). Expression of *bar*, the selective gene, yields phosphinothricin acetyl transferase (PAT) which inactivates the herbicides Basta and bialaphos by acetylation. The transformations also included one of two plasmids containing maize *kn1* (Vollbrecht et al., 1991). p35SAIKNN was constructed using a 1583 bp *Eco*RI fragment containing the *kn1* cDNA (Vollbrecht et al., 1991), ligated into the *Eco*RI site of a plasmid (pMF6) containing the cauliflower mosaic virus CaMV 35S (430 bp) promoter and the maize *adh1* intron 1 and *nos* terminator (Goff et al., 1990). pUbiUbiIKNN was constructed by sub-cloning the *ubiquitin* (*ubi1*) promoter and first intron from pAHC17 (Christensen et al., 1992) into pMF6 by removing the *Hin*dIII/*Bam*HI fragment (containing CaMV 35S/*adh1* intron 1) and inserting a *Hin*dIII/*Bam*HI fragment from pAHC17 containing the *ubi1* promoter and first intron; the 1518 bp *Bam*HI fragment containing the *kn1* cDNA was subsequently ligated into the *Bam*HI site of the constructed vector.

Transformation and regeneration of barley

Immature embryos (1.5-2.0 mm in length) from the barley cultivar Golden Promise were isolated and stably transformed using published methods (Lemaux et al., 1996; Wan and Lemaux, 1994). Briefly, one day before bombardment, immature embryos were cut in half longitudinally and placed scutellum-side up on callus-induction medium (Wan and Lemaux, 1994). Barley cells were transformed by microprojectile bombardment with a PDS1000He device (Bio-Rad, Hercules CA) at 1100 psi using 1 μm gold particles (Analytical Scientific Instruments, Richmond, CA) coated with 12.5 μg of pAHC20 plasmid and 12.5 μg of either p35SAIKNN or pUbiUbiIKNN. One day following bombardment, embryos were transferred to callus-induction medium containing the selective agent bialaphos (5 mg/l). Putatively transformed tissue was maintained, transferred to regeneration medium [FHG medium (Kasha et al., 1990) with 1 mg/l bialaphos] and incubated at 24°C in the light. Regenerated green plants (T_0) were transferred to soil and placed in the greenhouse. Five lines were obtained that regenerated fertile green Basta-resistant plants.

Molecular analysis

Genomic DNA was isolated from barley leaf and callus, and PCR reactions (50 μl) were performed on samples containing 0.5 μg of DNA. The primer pairs used for *bar* were Bar5F (5′ - C ATC GAG ACA AGC ACG GTC AAC TTC - 3′) and Bar1R (5′ - ATA TCC GAG CGC CTC GTG CAT GCG - 3′). The two primer pairs used for the barley *kn1* homologue were hvknox3B (5′ - CCC CTC AAA GTT CAG GTC AAT CCT - 3′) and hvknox3C (5′ - ACA TCA ATC AGA AAA GAA AGC ATT - 3′) and hvknox3B and hvknox3D (5′ - ATA AAA CCA GAA GAG TGT GGA GTA - 3′). PCR was performed using a PTC-100 Thermal Controller (MJ Research Inc., Watertown, MA) and PCR products were analyzed by electrophoresis. For detection of *kn1*-containing sequences, DNA hybridization was performed essentially as described (Wan et al., 1992) using a ^{32}P-labeled 0.42 kb *Kpn*I-*Eco*RI fragment from the *kn1* cDNA in p35SAIKNN. Leaf tissue from five T_1 lines was analyzed by DNA blot hybridization for the presence of *kn1*. Genomic DNA was isolated from leaf tissue (Cone, 1989), digested with *Hin*dIII, and used in hybridizations with a *kn1*-specific probe. A 2.9-kb fragment was released from p35SAIKNN by *Hin*dIII digestion, containing the 35S promoter, *adh1* intron1, *kn1* cDNA, and the *nos* 3′ end; from pUbiUbiIKNN a 3.8-kb fragment was released containing the *ubi1* promoter and intron, *kn1* cDNA and *nos* 3′ end. *kn1*-hybridizing sequences were detected from four of the five Basta-resistant lines and the patterns of hybridization were unique confirming that each line was derived from an independent insertion event.

Scanning electron microscopy

Fresh tissue was fixed in 3.7% formaldehyde, 45% ethanol, 5% glacial acetic acid and 1% Triton X-100 for 2 days at 4°C with gentle mixing, dehydrated in a graded ascending series of ethanol (70%, 80%, 90%, 95%, and 3 times with 100%) and critical point dried in a Samdri-PVT-3B (Tousimis Research Corp., Rockville, MD) in a CO_2 atmosphere. The samples were mounted on aluminum stubs with colloidal silver paste (TED Pella Inc., Redding, CA), coated with 25 nm gold from a Polaron E5400 sputter coater, and observed on an ISIDS-130 SEM equipped with a tungsten filament operated at an accelerating voltage of 10 kV (Bozzola and Russell, 1992).

Sectioning of plant tissues, immunohistochemistry and RNA in situ

Fixing and embedding of plant tissues followed published methods (Jackson, 1991). Briefly, samples from immature barley florets were fixed in 4% formaldehyde solution on ice, infiltrated under vacuum for 30 minutes, reimmersed in fixing solution at 4°C overnight and embedded in paraffin. Ten μm-thick paraffin sections were mounted on ProbeOn™ Plus microscope slides (FisherBiotech) and stored at 4°C. KN1 protein immunolocalization was performed following the protocol described for KN1 immunolocalization in maize using affinity-purified rabbit anti-KN1 antiserum (Lucas et al., 1995). In situ analysis of mRNA was performed using previously published methods (Jackson et al., 1994). The *kn1* probe consisted of three subclones (0.4-0.65 kb) of the 1.6 kb cDNA clone, transcribed separately and used as a mixture (Jackson et al., 1994).

Phenotypic analyses

Measurements were performed on segregating T_1 plants from line UbiKN-6. The five tallest tillers were removed from 6 mature plants containing *kn1* and from 4 negative segregants; measurements were performed on every tiller. Leaf length, determined on the last leaf to emerge before the flag leaf, was measured from the edge of the ligule to the tip of the leaf. To measure internode lengths, all leaves from each tiller were removed and each internode was independently measured from the base of the plant up to and including the last internode before the inflorescence. Tiller length was calculated as the summation of each internode length plus the length of the inflorescence. Phenotypic analyses of florets from wild-type barley and the *Hooded* varieties Strip Tease, Haybet and BGS152 were performed under a dissecting microscope; ten florets from five individual inflorescences from the same plant for a total of fifty florets were analyzed per *Hooded* variety. Scoring of the *Hooded* varieties for ectopic organ formation was based on observations of the presence of extra floral structures.

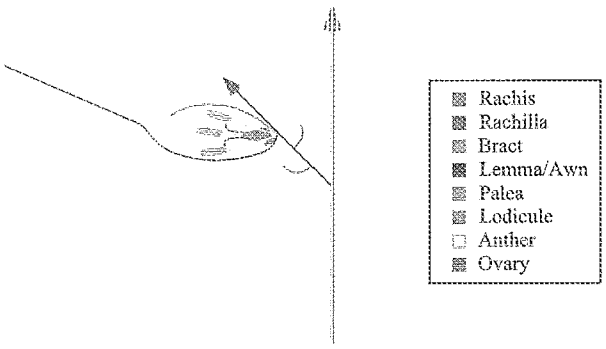

Fig. 1. Depiction of the barley spikelet. In barley, the inflorescence is composed of repeating units of spikelets which are attached to the main axis or rachis. The spikelet consists of a shortened stem axis, the rachilla, which bears overlapping bracts in a distichous order. The two proximal bracts of the spikelet do not develop flowers in their axils but remain as sterile or subtending glumes. The third bract, the lemma, is fertile. The lemma terminates in a long pointed extension, an awn. The floral axis originates in the axil of the lemma and bears the palea, two lodicules, three stamens and a pistil with a one-ovulate ovary.

RESULTS

The maize *kn1* gene affects plant height

We introduced the maize *kn1* gene into barley by stable transformation in order to assess the effect of ectopic expression on plant phenotype. Two plasmids were used for transformation, one containing a selectable marker and the other containing the maize *kn1* cDNA driven by either 35S/*adh1* intron (p35SAIKNN) or *ubiquitin/ubiquitin* intron (pUbiUbiIKNN). To confirm the presence of the transgene, progeny of the regenerated plants (T_1) from two p35SAIKNN lines (35SKN-1, -4) and two pUbiUbiIKNN lines (UbiKN-4, -6) were analyzed by DNA blot hybridization (data not shown). Two lines (35SKN-1, -4) contained the expected 2.9-kb fragment and one line (UbiKN-6) contained the expected 3.8-kb fragment. One line (UbiKN-4) did not contain an intact copy of the *kn1* gene.

T_1 plants from 35SKN-1 and -4 and UbiKN-6 were characterized phenotypically by comparing transgenic T_1 plants to their null segregants. Only one of the three transformed lines (UbiKN-6) exhibited measurable alterations in phenotype; the two 35S promoter-driven lines resembled wild-type plants. One of the most noticeable phenotypic changes observed in line UbiKN-6 was a reduction in overall plant height in the transgenic segregants (Fig. 2A), reflected in the shorter average tiller lengths for *kn1* segregants (38.30±4.08 cm, $P>0.995$), versus negative segregants (48.85±4.53 cm; Fig. 2B). The reduced tiller lengths were not due to a reduction in one or a subset of internodes but to a trend toward the reduction in all internode lengths (data not shown). Average leaf lengths of *kn1* segregants (19.61±2.45 cm, $P<0.55$), were not significantly different from leaf lengths of negative segregants (19.55±3.22 cm).

kn1 induces ectopic meristems

Florets from line UbiKN-6 showed major morphogenic alterations along the entire length of the inflorescence (Fig. 2C), the lemma and awn region being specifically affected. Relative to the wild-type floret (Fig. 3A) the transgenic florets often had outgrowths of tissue at the lemma/awn transition zone (Fig. 3D) or more distally toward the apex of the awn (Fig. 3B,C). When ectopic outgrowths were observed more distally along the awn, they protruded from both the edge of the margin as well as regions away from the margin and closer to the midrib (data not shown). Outgrowths that formed at the margins tended to be very hairy and disrupted the development at the edges of the awn as shown by SEM analyses (Fig. 3B,C). Outgrowths that occurred at the lemma/awn transition zone often produced lateral appendages, the shape of which was triangular (Fig. 3D). The orientation of this appendage was inverted 180 degrees relative to the primary floret as evidenced by the direction of epidermal hairs (Fig. 3D).

In the most severely affected floral structures of UbiKN-6, ectopic florets initiated from the adaxial surface near the lemma/awn transition zone (Fig. 3E-G). When ectopic immature florets were examined by SEM, floral primordia enclosed within the ectopic palea frequently had an incorrect number of floral organs and were abnormally developed; however, even in mature florets, the ectopic floral organs remained under-developed. The orientation of the first ectopic floret could be determined by the position of its palea (identified by its two major veins) which was inverted relative to the

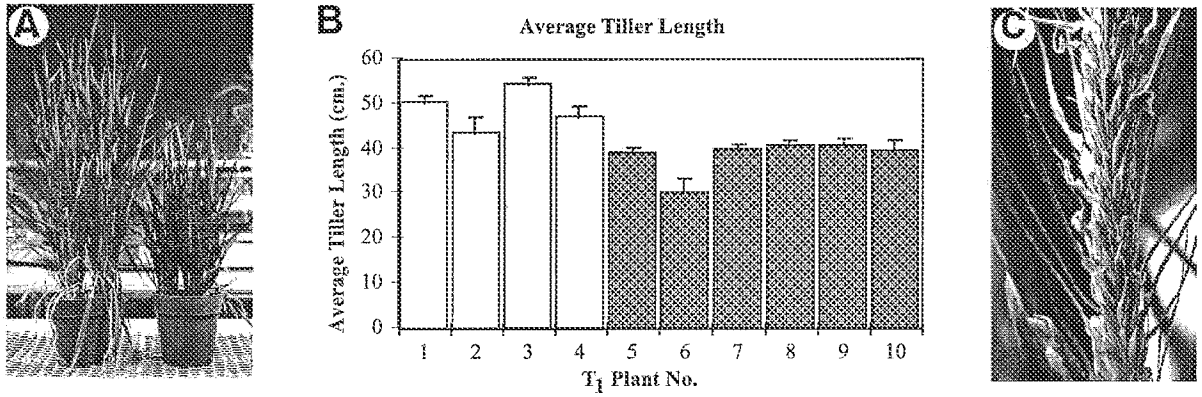

Fig. 2. Phenotypic variation in line UbiKN-6. (A) T_1-generation segregating plants (two plants per pot). The plant on the right contains the *kn1*-gene, the plant on the left a negative segregant. (B) Average tiller lengths of four negatively segregating plants (white) and six *kn1*-containing plants (shaded). (C) An inflorescence from line UbiKN-6.

orientation of the primary floret (Fig. 3E). The second ectopic floret was inverted relative to the first, such that the directionality of the second ectopic floret was the same as that of the primary floret (Fig. 3F,G). Perturbances in the lemma and awn were highly variable, from no visible aberration to multiple ectopic florets, with no apparent pattern in the severity along the inflorescence.

Molecular and phenotypic analysis of *Hooded* (*KK*)

Because of the similarities between *Hooded* and *kn1*-containing transgenic barley inflorescences, individual florets from plants bearing the *Hooded* mutation were scored for awn morphology and the presence of additional lemma, palea, anthers, ovaries or entire floral structures (Table 1) and characterized by SEM analysis. This analysis was performed on plants bearing *Hooded* in three different backgrounds and grown under identical, controlled growth conditions, to minimize the known effects of environmental conditions on the phenotype (Yagil, 1960; Yagil and Stebbins, 1969). To minimize the possibility that variability was due to different *Hooded* alleles in the three backgrounds, PCR analyses were performed. This analysis showed that all three *Hooded* mutants contain an approximate 300 bp insertion in intron 4 (data not shown), suggesting that all mutants likely derived from the same allele as described by Müller (Müller et al., 1995).

In Strip Tease (CI 6837), which contains *Hooded* in a six-rowed dwarf background, the hood remained rudimentary (Table 1); awns were often completely absent and small triangular-shaped appendages developed at the lemma/awn border (data not shown). When immature florets were examined by SEM, anther and ovule primordia were often observed enclosed within the ectopic palea and were frequently present in an incorrect number and were under-developed (Fig. 3H).

The six-rowed stock, BGS152, developed inflorescences with the most extreme *Hooded* phenotype (Table 1). The ectopic florets were nearly as fully developed morphologically as the primary florets. BGS152 florets, examined by SEM, were found to have two lateral triangular-shaped appendages between the unchanged lower portion of the lemma and the hood (data not shown) and resembled the lateral appendages produced at the lemma/awn border of UbiKN-6 transformants (Fig. 3D). In this region, a transition zone was observed at the

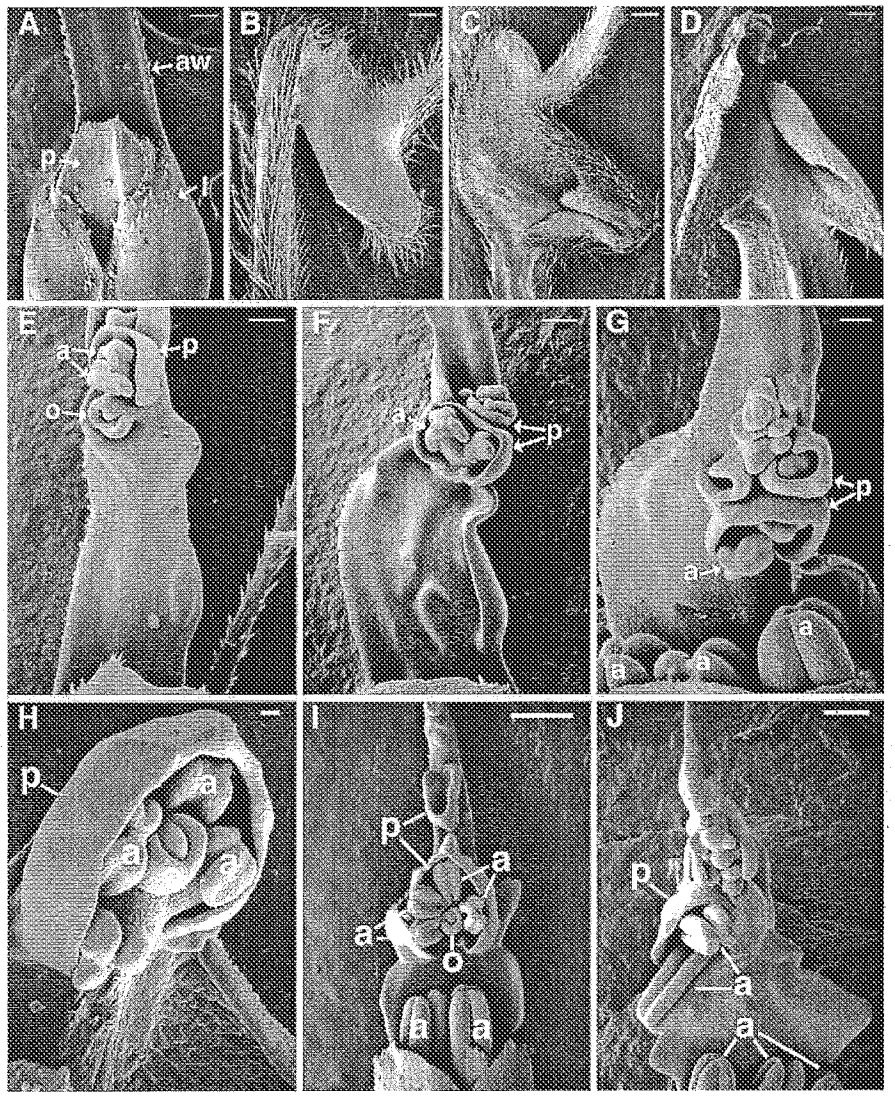

Fig. 3. Scanning electron micrographs of the adaxial surface along the awn. (A) Wild-type floret; (B-G) UbiKN-6 transgenic florets and (H-J) florets from the *Hooded* mutation in three different genetic backgrounds. (A) Wild-type floret at the lemma/awn border. (B) Adaxial view of an ectopic outgrowth of tissue on the awn. (C) An ectopic outgrowth with a partially inverted orientation. (D) An ectopic outgrowth in which the hairs resemble those found on the lemma and are inverted relative to the lemma hairs on the primary floret. (E) An ectopic floret on the awn consisting of a palea, anther and ovary (the tip of the palea from the primary floret is visible at the bottom of the photo). (F) Two ectopic florets, the first consisting of a palea and a single anther, the second, in opposite orientation to the first, consisting of a palea and rudimentary floral organ primordia. (G) Numerous ectopic meristems visible at the lemma/awn transition zone. (H) *Hooded* mutant Strip Tease. A single ectopic floret consisting of a palea, three anthers, and an ovary is shown. (I) *Hooded* mutant BGS152. The palea was removed to visualize the ectopic floret. The first ectopic floret contains a palea, four anthers and a single ovary. A second ectopic palea is in opposite orientation relative to the first ectopic floret. Additional meristem and palea primordia are visible more distally along the awn. (J) *Hooded* mutant Haybet. The first ectopic floret consists of a single palea and two anthers. Additional floral primordia are visible more distally along the lemma/awn. l, lemma; p, palea; aw, awn; a, anther; o, ovary. (Scale bar: A, 220 μm; B, 200 μm; C, 360 μm; D, 270 μm; E, 220 μm; F, 250 μm; G, 150 μm; H, 65 μm; I, 220 μm; J, 160 μm).

Table 1. Analysis of ectopic organ formation of *Hooded* in three backgrounds

	Lemma with a reduced awn	Lemma, rudimentary anthers and/or ovary (no-palea)	Lemma and rudimentary palea, only	1st*: entire flower	1st: entire flower 2nd†: lemma and palea only	1st: entire flower 2nd: lemma, palea rudimentary anther and/or ovary
BGS152				++++	++++‡	+
Strip Tease	++	++	++	++++§		
Haybet		+	++++	++++	+	+

+ = ~10%, ++ = ~20%, +++ = ~30%, ++++ = ~40% of flowers showing that phenotype per plant.
*First ectopic flower.
†Second ectopic flower.
‡All flowers had a reduced awn with multiple flaps of tissue progressing up the awn corresponding to successive palea primordia.
§Palea, anthers and ovaries were always underdeveloped.

lemma/hood border demarcating the junction between two lemmas. The inversion of the first ectopic floret relative to the primary floret was documented by examining the direction of the hairs on the epidermal surface of the ectopic lemma and by the orientation of the ectopic palea (Fig. 3I). Multiple floral primordia were observed forming along the entire length of the awn (Fig. 3I) with each primordia inverting its orientation relative to the preceding primordia.

'Haybet', which contains the *Hooded* mutation in the two-rowed Betzes background, developed the greatest array of *Hooded* phenotypes (Table 1). In the mildest cases, only very rudimentary floral organs formed; in the more severe instances, fully developed secondary florets formed. Often, the ectopic florets would develop abnormally producing ectopic floral organs containing large numbers of anthers (data not shown). Closer examination of immature florets revealed that multiple floral primordia were initiating along the adaxial surface of the awn (Fig. 3J) although not in as orderly a manner as in BGS152. In the most severe cases, a second ectopic floret formed with the full complement of floral structures and in the opposite orientation to the first ectopic floret, as was observed in BGS152.

Expression analysis

The barley HVKNOX3 protein is 90% identical to KN1 (Müller et al., 1995). Because of this similarity we were able to use the antibody to KN1 (Smith et al., 1992) in protein immunolocalization studies on immature inflorescences of *Hooded* mutants as well as transgenic and null segregants of 35SKN-1 and UbiKN-6. In serial sections through the inflorescence of negative segregants, accumulation of high levels of endogenous KN1 cross-reacting proteins were observed in immature vascular bundles within the floral shoot axis and more weakly in the ground tissue of the inflorescence shoot (Fig. 4A). Expression was also observed in rachilla (Fig. 4A) which give rise to the floret and in immature floral meristems (data not shown). No expression was observed in lateral organs such as lemma/awn, palea, anther and ovary (Fig. 4A). No antibody binding was seen in sections labeled with mock affinity-purified pre-immune serum (data not shown).

Immunolocalization was also performed on *kn1*-containing segregants of lines 35SKN-1 and UbiKN-6. No ectopic expression was observed in the lemma/awn of florets from 35SKN-1 (data not shown). In the UbiKN-6 line, the expression pattern through a median longitudinal section of an immature inflorescence was nearly identical to the negative segregant (Fig. 4A), with the exception of a small region of ectopic expression on the lemma/awn border (Fig. 4B). This zone of expression was not observed on every lemma along an inflorescence but appeared sporadically as did the ectopic inflorescences. Young florets from line UbiKN-6 that have ectopic florets initiating along the lemma show expression in epidermal and subepidermal layers on both the adaxial and abaxial surfaces of the lemma in the region proximal to the ectopic floret (Fig. 4C). This expression terminates on the abaxial side at a position directly below the ectopic floret; however, on the adaxial surface the expression continues more distally along the lemma (Fig. 4C,D). We observed expression in all cells that comprise the base of the ectopic floret. Expression was observed in the cells which comprise the base of the filament of the immature ectopic stamen and also in the epidermal layer of the anther but was absent from the ectopic palea (Fig. 4C,D).

In immunolocalization studies on immature florets from the *Hooded* mutant BGS152, expression patterns were found to be nearly identical to those observed in florets from line UbiKN-6 with two exceptions. First, we did not observe expression in the epidermal cells on either the adaxial or abaxial side of the lemma proximal to the ectopic flower (Fig. 4E). Secondly, in the *Hooded* varieties expression extended from the ectopic floret distally toward the apex of the awn and was observed in the additional meristems initiating along the awn (Fig. 4F).

Analysis of mRNA by in situ hybridization was performed on immature inflorescences and floral tissues of transgenic and null segregants of 35SKN-1 and UbiKN-6. In serial sections through the inflorescence of negative segregants, significant accumulation of *kn1* mRNA was not detected. No ectopic *kn1* transcript was observed in the lemma/awn of florets from 35SKN-1 (data not shown). However, in 8-9 serial sections through the inflorescence of UbiKN-6, a small region of *kn1* mRNA accumulation was detected in the lemmas of some of the florets (Fig. 4G). The expression was also detected in the base of the ectopic floret as well as in the ectopic floral organs (Fig. 4H).

DISCUSSION

In this paper we describe the creation of transgenic barley plants expressing the maize homeobox gene *knotted1* (*kn1*). The introduction and ectopic expression of *kn1* in barley phenocopies the naturally occurring dominant mutant, *Hooded*, in which the awn is triggered to form a series of ectopic floral meristems. This is consistent with the idea that members of the

kn1 gene family function in meristems (Kerstetter et al., 1997; Long et al., 1996; Sinha et al., 1993; Smith et al., 1992).

Phenotypic analyses of transgenic *kn1*-expressing barley and *Hooded*

Analysis of our data shows that ectopic expression of *kn1* is sufficient to induce development of ectopic florets on the adaxial surface of the lemma/awn of barley. Intermediate phenotypes include various types and amounts of ectopic outgrowths of tissue near the lemma/awn transition zone and more distally along the awn (Fig. 3B-D). SEM analysis of immature transgenic inflorescences revealed that, when ectopic florets developed, they were located at the distal end of the lemma and remained under-developed. They also formed in an inverse orientation to the preceding floret (Fig. 3F,G), similar to the situation seen in *Hooded* mutant florets (Fig. 3I,J). The differences observed between the three *Hooded* mutant florets (Fig. 3H-J) suggests that different backgrounds have noticeable effects on the severity and appearance of the mutant phenotype between different genotypes. Therefore, it is likely that the severity and nature of the phenotype of the *kn1*-expressing plants were influenced by the genetic background.

We also observed an overall reduction in plant height in transgenic barley plants (Fig. 2A) similar to that previously

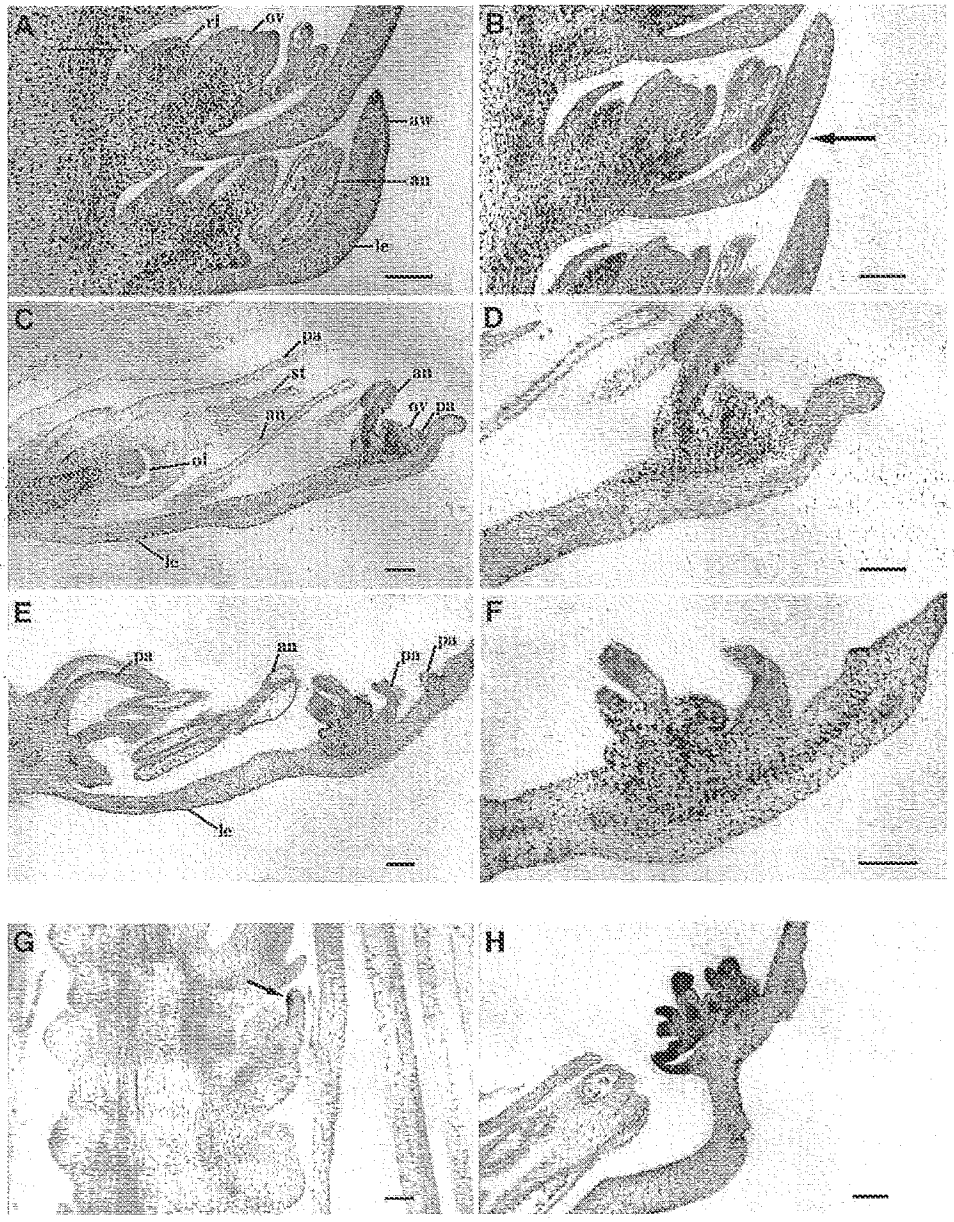

Fig. 4. In situ immunohistochemical analysis using antibody to KN1 (A-F) (Lucas et al., 1995) and mRNA hybridization analysis using *kn1* cDNA as a probe (G and H)(Jackson et al., 1994). (A) Florets on an immature inflorescence of wild-type barley. Expression is restricted to stem portions of the rachis and to the rachilla; no expression is detected in differentiated organs, e.g. lemma, awn, anther, and ovary. (B) Florets on an immature inflorescence of UbiKN-6 transformant; expression is also seen in the rachis and rachilla. In the region of the lemma/awn transition zone, localized ectopic expression is observed in the region that is presumed to become the ectopic floret (arrow). (C) An individual floret of line UbiKN-6. The primary floret is at the left. The staining at the base of the ovule is in the normal zone of expression; however, ectopic expression is observed in the epidermal/subepidermal cell layers on both the adaxial and abaxial surfaces of the lemma at the position of an ectopic floret as well as in the ectopic floret. (D) A closer view of the ectopic floret in C. Expression is observed in the base of the filament of the immature ectopic stamen and also in the epidermal/subepidermal layer of the anther but was absent from the ectopic palea. (E) An individual floret of *Hooded*. The primary floret palea, anther and ovary is at the left; normal zones of expression are observed at the base of the floret. Expression patterns are nearly identical to those observed in florets from line UbiKN-6; however, expression is not observed in the epidermal/subepidermal cells on either the adaxial or abaxial side of the lemma. (F) A closer view of the ectopic floret in E. (G) Florets on an immature inflorescence of UbiKN-6 transformant. In the region of the lemma/awn transition zone, localized ectopic *kn1* mRNA is observed in the region that is presumed to become the ectopic floret (arrow). (H) An individual lemma from a floret of line UbiKN-6. The anther from the primary floret is at the left. *kn1* mRNA is at the base of the ectopic florets and extends up into the developing ectopic floral organs. rc, rachis; rl, rachilla; le, lemma; aw, awn; pa, palea; an, anther; ov, ovary; st, stigma; ol, ovule. Scale bars: 50 μm.

observed in transgenic maize, *Arabidopsis*, tomato and tobacco expressing *kn1* driven by a constitutive promoter (R. Williams-Carrier unpublished data; Chuck et al., 1996; Hareven et al., 1996; Sinha et al., 1993, respectively). Severe alleles of *Kn1* in maize also show a reduction in plant height (Veit et al., 1990) although no effect of plant height was observed in the *Hooded* backgrounds studied. It has been proposed that the dwarfing effect of *Kn1* results from a reduction in the rate of cell divisions (Sinha et al., 1993).

Differing responses of monocot and dicot tissues to KN1 expression

Expression of *kn1* and *kn1*-like genes in dicotyledonous species has caused leaf phenotypes dramatically different from the phenotypes observed when the same or similar constructs were expressed in monocots. Tobacco plants transformed with the *kn1* cDNA produce leaves that are diminutive with numerous ectopic shoot meristems initiating across the adaxial surface of the leaves (Sinha et al., 1993). A similar situation was observed in *Arabidopsis* when the *kn1*-like gene, *KNAT1*, was expressed in transgenic *Arabidopsis* (Chuck et al., 1996; Lincoln et al., 1994). Ectopic expression converted simple leaves into lobed leaves and ectopic meristems developed in the sinus region of the lobes. In both tobacco and *Arabidopsis*, vegetative meristems developed on vegetative leaves while inflorescence meristems developed on leaves that subtended florets, i.e. the developmental phase of the plant determined the identity of the meristem.

In contrast, the production of meristems on vegetative leaves was never observed in monocots. In transgenic maize only vein clearing, knots and ligule displacement occurred (R. Williams-Carrier, unpublished data), a situation similar to that seen in the maize dominant *Kn1* mutants (Freeling and Hake, 1985). In transgenic barley line UbiKN-6 no leaf phenotypes were observed; although, more recently isolated lines are displaying knots on leaves (R. Williams-Carrier, unpublished data). Transformation of rice with the *kn1*-like gene *OSH1* (Matsuoka et al., 1993) also resulted in knots forming on the leaves but no ectopic meristems. The fact that monocots ectopically expressing *kn1* and *kn1*-like genes do not produce meristems from their leaves, as seen in tobacco and *Arabidopsis*, may reflect a fundamental difference in the plasticity of monocot and dicot leaf cells. Leaf cells of dicots may retain a more flexible, less-determined state allowing them to respond to signals in a different manner. This idea is consistent with the observation that dicot leaf tissue can be manipulated in vitro to produce totipotent tissue that gives rise to fertile plants whereas cultured monocot leaf cells seldom are reported to give rise to plants (Potrykus, 1980).

Since monocot leaves are unable to produce ectopic meristems, we are left with the question of why the barley awn, considered to be a modified leaf blade (Dahlgren et al., 1985), produces ectopic meristems. Previous histological data showed that mitotic spindles in wild-type awn cells are consistently oriented parallel to the long axis of the awn and all divisions in the epidermal and subepidermal layers are anticlinal (Stebbins and Yagil, 1966). In *Hooded* mutants, however, the epidermal cells in the region of the developing ectopic florets divide at various angles with respect to the long axis of the lemma. Periclinal divisions occur in the subepidermal layers

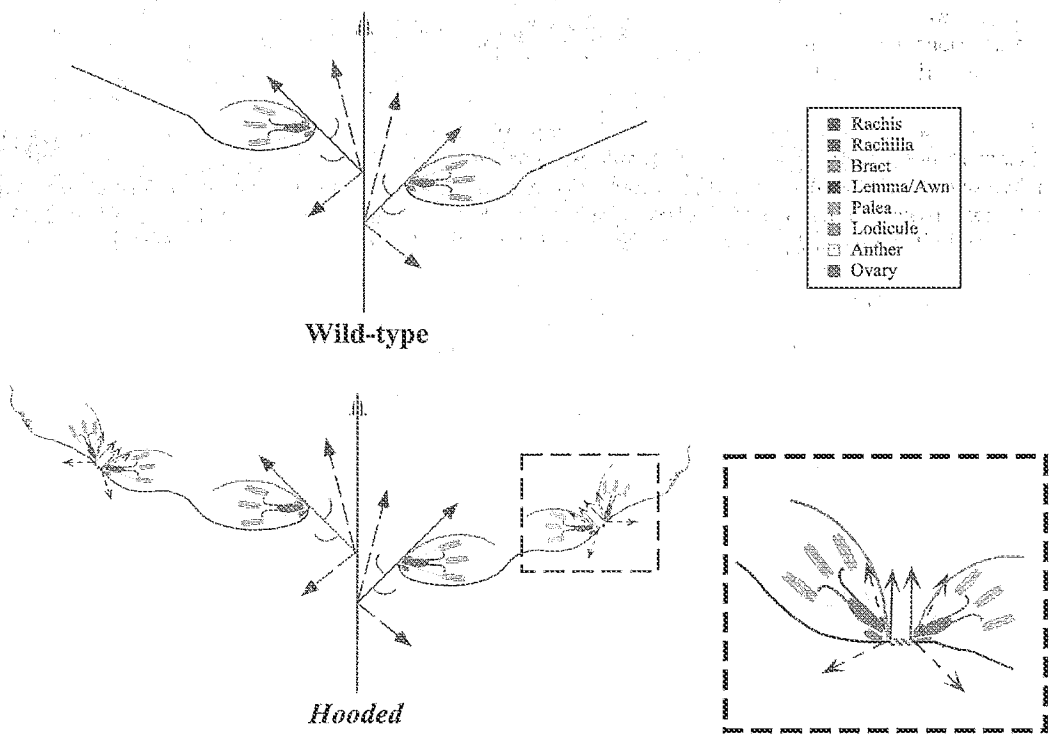

Fig 5. Model to explain the morphology of wild-type versus *Hooded* mutant barley inflorescences. The wild-type barley inflorescence (top) is composed of repeating units of spikelets which are attached to the main rachis; three spikelets arise at each node along the inflorescence. The *Hooded* inflorescence pattern as specified by ectopic expression of *hvknox3* has ectopic flowers along the awn (bottom). Our model suggests that the ectopic florets are actually products of additional inflorescence meristems (red-stripe) that form along the lemma/awn (dark green). In *Hooded*, ectopically produced inflorescence meristems initiate spikelets that mimic the same pattern as that laid down by the wild-type inflorescence. Initiation of florets from the two central spikelets gives rise to the opposing florets observed in the *kn1*-transgenic and *Hooded* mutant plants. The reiteration of the opposing florets along the awn is proposed to be due to the establishment of additional regions of inflorescence identity along the awn.

and give rise to the elevated dome from which the organs of the first ectopic floret are differentiated (Stebbins and Yagil, 1966). Awn cells may lack the constraints in cell division patterns inherent in other monocot leaf cells.

Expression patterns of UbiKN-6 mimic those in *Hooded*

Many similarities were observed between the pattern of ectopic KN1 accumulation within the developing florets of the UbiKN-6 transgenic line compared to that of *hvknox3* in *Hooded* mutants (Fig. 4C,E and Müller et al., 1995). In both *Hooded* and UbiKN-6, the region of the awn that expresses KN1 appears to be the site of formation of the ectopic floret. Immunolocalization and mRNA hybridization analyses of the UbiKN-6 line revealed that a small zone of *kn1* protein and mRNA accumulates near the lemma/awn transition zone in immature florets (Fig. 4B,G); however, this expression was variable from floret to floret along an inflorescence.

Our finding that *kn1* mRNA and protein accumulation in UbiKN-6 was limited to a specific subset of cells that give rise to ectopic florets was unexpected since expression of the *kn1* cDNA was controlled by the *ubiquitin* promoter (Christensen and Quail, 1996), previously shown to confer constitutive expression in a variety of tissues in transgenic rice (Cornejo et al., 1993; Takimoto et al., 1994). Histochemical GUS assays on immature barley awn tissues transformed with a *ubiquitin* promoter-driven *uidA* construct revealed uniform blue staining throughout all stages of the developing awn (R. Williams-Carrier, unpublished results) demonstrating that the *ubiquitin* promoter drives constitutive expression throughout this organ. In 35SKN-1 transformants, in which no phenotypic variation was observed in the awns, no ectopic expression of *kn1* was detected. It is possible in barley that levels of expression of transgenes driven by the 35S promoter generally are lower than when driven by the *ubiquitin* promoter (Schledzewski and Mendel, 1994; M.-J. Cho and P. G. Lemaux, unpublished data); however, this does not explain the localized expression observed in UbiKN-6.

We propose that *kn1* is regulated posttranscriptionally in these transgenic plants. Similar regulation may explain the expression patterns seen in 35S:KNAT1 *Arabidopsis* plants (Chuck et al., 1996). Although it is possible that ectopic KN1 expression induces *hvknox3* expression in the ectopic meristem, the *kn1* mRNA hybridization data does not support such a model. The regulated expression observed in the awn suggests that these cells are unique, not only in their ability to divide in different planes but also in their capacity to express and/or respond to KN1. It is likely that *kn1* expression requires the cooperation of, or is inhibited by, other factors that are expressed in a spatially or temporally restricted pattern during development.

A model for ectopic inflorescence meristem formation

One of the more striking features of the meristems formed on awns is their iterative pattern of reverse orientation, a finding that has puzzled former investigators (for review, Stebbins and Yagil, 1966). Stebbins observed that the cellular patterning of the meristematic dome resembles the portion of the rachis from which spikelets differentiate. We propose a model to explain this phenomenon based on the KN1-induced transformation of the barley awn to an inflorescence meristem (Fig. 5), rather than simply to a floral meristem. In barley, three spikelets arise at each node along the inflorescence. In two-rowed barley, the central spikelets are fertile and the lateral spikelets remain reduced. In the ectopically expressing *kn1*-transgenic and *hvknox3 Hooded* mutant plants, accumulation of KN1 and KNOX3 signals cells to initiate inflorescence meristems on the awn creating zones of rachis identity. The ectopically produced inflorescence meristems then initiate spikelets that mimic the same pattern as that laid down by the wild-type inflorescence; the two spikelets oppose each other in orientation. Normally, in a six-rowed variety three spikelets develop at each node of an inflorescence; however, in this instance, only the two central spikelets, directly opposite each other, develop (Fig. 1). This may be due to a limitation in the number of cells that can be recruited to form ectopic inflorescences or localized space constraints limiting the development of the inflorescence. The two central spikelets initiate florets which give rise to the opposing florets observed in the transgenic and mutant plants. The reiteration of the opposing florets along the awn is due to the establishment of additional regions of inflorescence identity along the awn.

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